

# Overproduction of Mercuric Reductase Protein Expressed by Synthetic merA Gene and Reduction of Inorganic Mercury HgCl<sub>2</sub> *by Billy Kepel 3*

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## Overproduction of mercuric reductase protein expressed by synthetic *merA* gene and reduction of inorganic mercury $HgCl_2$

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Mercury, recognized as one of the most toxic heavy metals in the environment, exists in three forms: elemental mercury, inorganic mercury compounds (primarily mercuric chloride), and organic mercury compounds (primarily methyl mercury). Inorganic mercury can be detoxified using mercuric reductase (*MerA*). In our previous study, mercuric reductase gene had been isolated from mercuric resistant bacterium *Klebsiella pneumoniae*. The bacterium was obtained from Sario river estuary. This study aimed to analyze the ability of *merA* protein resulted from transformation and overproduction in reducing inorganic mercury  $HgCl_2$ . The *merA* gene encoding for *MerA* protein with the length of 571 aa was synthesized and cloned into expression vector pJexpress414. The vector was transformed into *E. coli* Top-10. The *merA* gene was reisolated, sequenced and characterized. Overproduction of *MerA* protein in *E. coli* BL-21 was induced using IPTG to produce the protein in higher amount. The resulting protein was purified and characterized. The protein was tested for its ability to reduce inorganic mercury. Into *MerA* protein solution,  $HgCl_2$  10 ppm, NADH 0.1 mM, and FAD 0.1 mM were added and the solution was incubated for 24 hours. The results were compared with *MerA* recombinant produced by *E. coli* BL-21(DE3) in reducing inorganic mercury. The transformed *merA* gene has a length of 1695 bp encoding for 571 amino acid. The overproduced *MerA* protein induced by IPTG 0.1 mM has a size of 63 kDa at 37°C. The ability of recombinant *MerA* protein and mercury reductase resulting from overproduction to reduce  $HgCl_2$  were 100% and 80%, respectively. This research concluded that overproduced *MerA* protein isolated from mercuric resistant bacteria *K. pneumoniae* was able to detoxify inorganic mercury.

**Keywords:** overproduction, *MerA* protein, *merA* gene, mercuric reductase, inorganic mercury.

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### INTRODUCTION

The release of heavy metals into the environment can harm the ecosystem and cause serious harm to human health. Mercury is one of the most dangerous heavy metals. In very small amounts, mercury can have a neurotoxic effect (Rasmussen et al, 2008). Toxic effects of mercury can also cause birth defects, as well as problems with the gastrointestinal and urinary tract (Dimer, 2007). The Minamata case of Japan in 1950 and in Iraq 1971-1972 are examples of the neurotoxic effects of poisoning by consuming methyl mercury

contaminated foods (Al-Madani et al, 2009). It is currently predicted that more than 1500 tons of hazardous mercury wastes are discharged into the environment each year in Asia and Africa. Continuous mercury pollution is increasing over time due to human activity, such as the rise of the electronics industry, antimicrobials, vaccines, amalgam, cosmetics and mercury gold mines (Jan et al, 2009; Schelert et al, 2003).

Wasted mercury accumulates in soil and water as toxic  $Hg^{2+}$  mercury ions, which then by microbes it can be changed into more toxic

methylmercury. Humans may be exposed to certain types of mercury, including mercury ( $\text{Hg}^+$ , which exists as  $\text{Hg}_2^{2+}$ ) ions, mercury ion ( $\text{Hg}^{2+}$ ), trace element mercury ( $\text{Hg}^0$ ), and Cinnabar, a naturally occurring mercuric sulfide ( $\text{HgS}$ ) (Schelert et al, 2003). Exposure to mercury ion and organomercury will have an effect on the central nervous system, while inorganic mercury affects the kidneys (Jan et al, 2009).

Various conventional techniques have been used to remove toxic metals including chemical preparation and separation, oxidation-reduction reactions, ion exchange, reverse osmosis, filtration, adsorption using activated carbon, electrochemistry and evaporation. However, all such methods have not been effective, especially for metallic concentrations less than 100 mg/l. These techniques are also quite expensive, and usually use other chemicals that can become secondary pollutants (Habashi, 1978). Therefore, the use of microorganisms to remove heavy metal contamination from mining and industrial waste becomes a necessary consideration (Perisa et al, 2011). Mercury chloride ( $\text{HgCl}_2$ ) is often used for research because it is easy to dissolve and is toxic (Schelert et al, 2003). Detoxification of mercury by microbes is by changing the form of mercury ion  $\text{Hg}^{2+}$  into trace element mercury ( $\text{Hg}^0$ ), which is less toxic and can evaporate. *Staphylococcus*, *Bacillus*, *Pseudomonas*, *Citrobacter*, *Klebsiella* and *Rhodococcus* are amongst the microbes often used in mercury bioremediation (Akiniji, 2004).

Detoxification of mercury by mercury resistant bacteria occurs because they have a mercury resistance (*mer*) operon. The *mer* operon involves in detoxifying organometallic or inorganic compounds. The *mer* operons are consisted of genes that encode the functional proteins for regulation (*merR*), transport (*merT*, *merP* and/or *merC*, *merF*) and reduction (*merA*) (Nascimento and Charó-Souza, 2003; Schelert et al, 2003). The additional *merB* genes are sometimes found downstream of the *merA* gene in the *mer* operon (Osborn et al., 1997). The *merA* gene encodes for mercuric reductase while *merB* gene encodes for organomercuric lyase. Mercuric lyase can convert organic mercury into inorganic mercury, and mercury reductase can catalyze the reaction of inorganic mercury ( $\text{Hg}^{2+}$ ) changes into volatile trace element mercury ( $\text{Hg}^0$ ). The reaction depends on NAD(P)H (Barkay and Wagner-Döbler, 2005). The volatile mercury which has been formed is fluxed out from cytosolic region into outer periplasm. Because this trace element

mercury is volatile, it can get out of bacterial cells itself. The use of mercuric reductase to solve the problem of inorganic mercury contamination in the environment is potentially very high. Bacteria that produce mercuric reductase and mercuric lyase genes have been widely exploited from the environment (Vetriani et al, 2005; Poulain et al, 2007; Ni Chadchain et al., 2006; Zeyaulah et al., 2010; Fatimawali et al, 2011; Billy et. al, 2012).

Zeyaulah et al. (2010) isolated *E. coli* bacteria that were resistant to mercury from mercury-contaminated environment in India. They succeeded to clone and express *merA* gene in *E. coli* BL-21 and were able to obtain 66.2 kDa *MerA* protein. Fatimawali et al. (2011) isolated mercuric-resistant bacteria *Klebsiella pneumoniae* from an estuary in Manado. They were also able to purify *MerA* protein resulted from overproduction inside *E. coli* BL-21 induced with IPTG. On SDS PAGE, the *MerA* protein has size of 60 kDa. The use of mercuric reductase to overcome the problem of inorganic mercury contamination in the environment is potentially very high, therefore the process of cloning and expression of *merA* gene is very useful for obtaining *MerA* protein in significant amounts (Fatimawali et al, 2015). This research was conducted with the aim to transform, overproduce, and purify *MerA* protein, then test its ability to reduce inorganic mercury  $\text{HgCl}_2$ .

## MATERIALS AND METHODS

The following procedure was modified from Zeyaulah et al. (2010):

### Plasmid Elution

Approximately 2-5  $\mu\text{g}$  of synthesized plasmids containing *merA* gene from *K. pneumoniae* was dissolved in 50  $\mu\text{l}$  sterile aquabidest in an Eppendorf and centrifuged. One ml of rejuvenated culture of *E. coli* was added to 2 ml Eppendorf. There were 3 rejuvenated cultures each for *E. coli* Top-10 and BL-21, respectively. Each culture was centrifuged at 3670 rpm at 4°C for 5 minutes. The cultures were subsequently placed on ice for 10 minutes and centrifuged again for 1 minute. The supernatants were discarded. The procedure was repeated 5 times until the pellets from 5 ml bacterial culture were obtained. To each Eppendorf, 200  $\mu\text{l}$  TSS (transformation and storage solution) buffer was added and mixed until the pellet became diluted by inverting the tube several times.

### Transformation of *merA* gene

Into 200  $\mu$ l of TSS containing each of *E. coli* Top-10 and BL-21, 5  $\mu$ l of plasmids was added into respective tube and mixed gently. Each tube was incubated on ice for 30 minutes, and the cells were shocked by heating at 42°C for 10 seconds. The tubes were subsequently placed on ice for 5 minutes. Into each tube, 800  $\mu$ l of SOC medium was added into the mixture. Each tube was incubated with shaking for 60 minutes at 250 rpm at 37°C. The tube was centrifuged at low rpm and as much as 800  $\mu$ l of suspension was taken out and leaving 200  $\mu$ l. The cells were mixed thoroughly by flicking and inverting the tube. The selection plates were warmed to 37°C. Onto each plate, 50  $\mu$ l of each suspension was pipetted. There were three plates for *E. coli* Top-10, two plates contained ampicillin (A) and one plate without ampicillin (B), three plates for *E. coli* BL-21 (two plates contained ampicillin (C) and one plate without ampicillin (D)). The cells were distributed evenly on agar and incubated overnight at 17 hours.

### Quick lysis of recombinant *E. coli*

Each of recombinant cells on plate A, B, C, and D was picked using a sterile loop, placed into Eppendorf tube, respectively, and added with EDTA (10 mM, pH 8), added with lysis solution (2  $\mu$ l NaOH 5N, 50  $\mu$ l SDS 10%, 400  $\mu$ l sucrose 10%, ddH<sub>2</sub>O 548  $\mu$ l), vortexed for 30 seconds, then incubated on ice for 5 minutes. The solution was centrifuged at 12,000 rpm for three minutes and electrophoresed on 1% agar for 30-45 minutes in PCR buffer, visualized with UV illuminator to check DNA bands of 1695 kb for BL-21 (A) and Top-10 (C), and no band for B and D, indicating that the plasmid transformations on Top-10 and BL-21 was successful.

### Overproduction of *MerA* protein from recombinant *E. coli* BL-21

Recombinant *E. coli* BL-21 was cultured in Luria-Bertani medium containing ampicillin 100  $\mu$ g/ml until reaching log phase (OD 600 nm ~ 0.7) at 37°C, then induced with IPTG 0.5 mM for 4 hours. The cells were centrifuged and pellet was lysed, *MerA* protein was analysed on SDS-PAGE. The overproduction was successful if there was thick protein band with the size of 63 kDa.

### Optimization of *MerA* protein overproduction

After the best induction temperature was obtained, several final IPTG concentrations (0.1, 1.0, and 5.0 mM) were used in this optimization.

Overproduction optimization was conducted at the best induction temperature for 4 hours. Pellet was lysed and *MerA* protein was analyzed on SDS-PAGE. The best IPTG concentration was obtained if successful in producing thick band of protein 63kDa.

### Purification of *MerA* protein

Supernatant obtained from overproduction was transferred into polypropylene column containing Ni-NTA (nickel-nitrilotriacetic acid) and eluted with buffer containing imidazole with successive concentrations of 15, 50, 60, 70, 80, 90, 100, 130 and 250 mM. The fraction obtained was centrifuged at 1000 rpm for 2 minutes and supernatant was analyzed with SDS-PAGE for thick band of protein with the size of 63 kDa. The purification results were stored at 8°C for further testing of their activity to reduce inorganic mercury.

### Activity test of overproduced protein *MerA*

Activity test of recombinant protein *MerA* obtained from overproduction purification and recombinant *E. coli* BL-21 (C) was performed as follows:

### Activity test on recombinant *E. coli* BL-21

Into 100 ml of LB medium containing HgCl<sub>2</sub> ppm, FAD 0.1 mM and NAD(P)H 0.1 mM were added. Twenty ml solution was taken out as negative control (tube 1), and the remaining solution was added with 1  $\mu$ l of *E. coli* BL-21 (DE3) containing recombinant protein *MerA*, and divided into 4 tubes, each of 20 ml (tubes II-V). The tubes were incubated at 37°C for 24 hours. Afterwards, concentration of Hg was determined using CVAAS.

### Activity test on overproduced and purified *MerA*

NAD(P)H 0.1 mM and FAD 0.1 mM were added into 100 ml of buffer solution containing HgCl<sub>2</sub> 20 ppm. Twenty ml solution was set aside as negative control (tube VI). The remaining solution (80 ml) was added with *MerA* 5 mg, mixed thoroughly, and divided into 4 reaction tubes with 20 ml solution for each tube (tube VII-X). The tubes were incubated for 24 hours and the concentration of Hg was determined using CVAAS.

## RESULTS

Mercuric reductase gene used in this study was cloned previously into pJExpress414 by

Qiagen Korea. The length of the gene was 5707 bp harboring *merA* gene with the size of 1695 bp which was synthesized based on the size of *merA* gene of *Klebsiella pneumoniae* isolate A1.1.1

obtained from Sario estuary (Fatimawali et al, 2015), as seen in Figure 1.

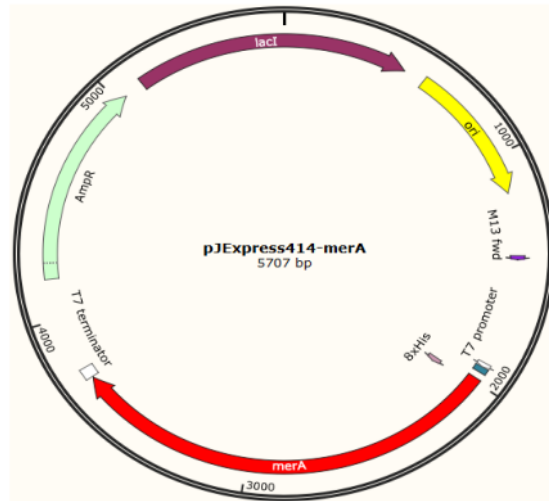


Figure 1. Plasmid pJExpress414 harboring *merA* gene encodes for mercuric reductase, fused with a his-tag, T7 promoter, and T7 terminator.

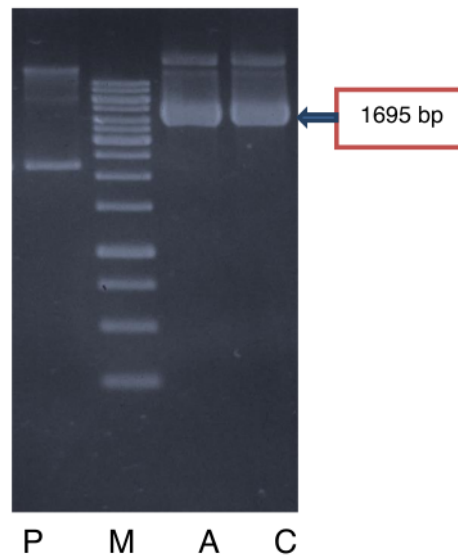
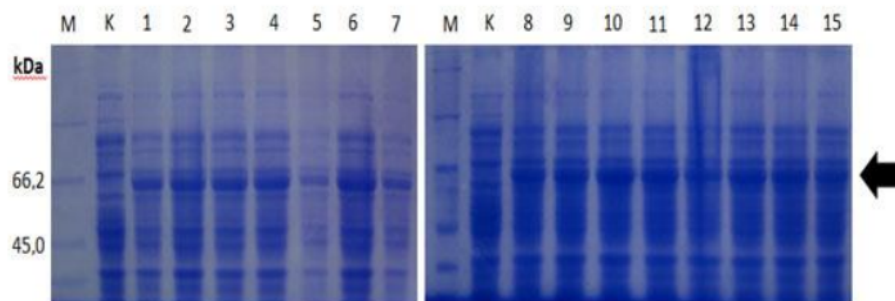
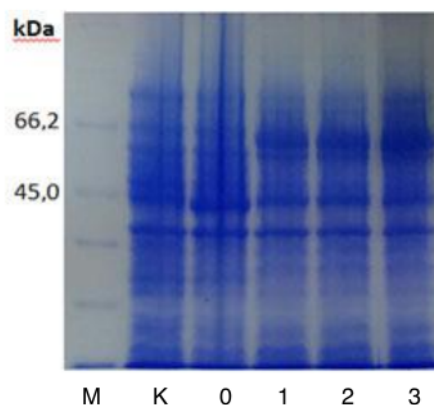


Figure 2. Electrophoresis of *merA* gene resulted from transformation of pJExpress414-*merA* into *E. coli* Top-10 and BL-21. (P) comparison, (M) marker, (A) *merA* from *E. coli* Top-10, (C) *merA* from *E. coli* BL-21.



33 **Figure 3. SDS-PAGE of overproduced recombinant *MerA* protein expressed in *E. coli* BL21 (DE3). (M) marker, (K) no plasmid, 1-15 = transformant.**



20 **Figure 4. Overproduction optimization of recombinant *MerA* protein induced with IPTG. (M) marker, (K) negative control, (0) IPTG 0 mM, (1) IPTG 0.1 mM, (2) IPTG 1.0 mM, (3) IPTG 5.0 mM).**

The result of overproduction optimization of recombinant *E. coli* BL21 (DE3) is shown in Figure 4. Of the three concentration of IPTG (0.1, 1.0, and 5.0 mM), the results were the same, therefore the lowest concentration of IPTG (0.1 mM) was used during subsequent overproduction. The overproduced *MerA* protein was sonicated for 9 minutes at amplitude of 60%, 15 seconds on-off pulse rate. The supernatant was purified using Ni-NTA column, eluted using increasing imidazole concentration (15, 25, 50, 75, 100, 125, 150, 200, and 250 mM). The targeted protein started to elute on imidazole fraction of 75 mM. The result is shown in Figure 5. The eluted pure *MerA* protein was collected and tested for its reduction power on inorganic mercury  $HgCl_2$ . The result is shown in Figure 6.

As seen on Table 1 and Figure 6, the mercury up to 99.72%, while overproduced mercuric reductase (*MerA*) protein on *E. coli* Top-10 could

reduce inorganic mercury up to 70.4% after incubation for 24 hours at 37°C recombinant *E. coli* BL21 (DE3) could reduce inorganic

**Table 1. The reduction of inorganic mercury  $HgCl_2$**

No.	Treatment	$HgCl_2$ (ppm)
Recombinant <i>MerA</i> protein		
1.	Tube I	18.12
2.	Tube II	0.04
3.	Tube III	0.05
4.	Tube IV	0.03
5.	Tube V	0.08
Overproduced <i>MerA</i> protein		
6	Tube I	19.16
7.	Tube II	6.08
8.	Tube III	5.26
9.	Tube IV	5.36
10.	Tube V	6.21

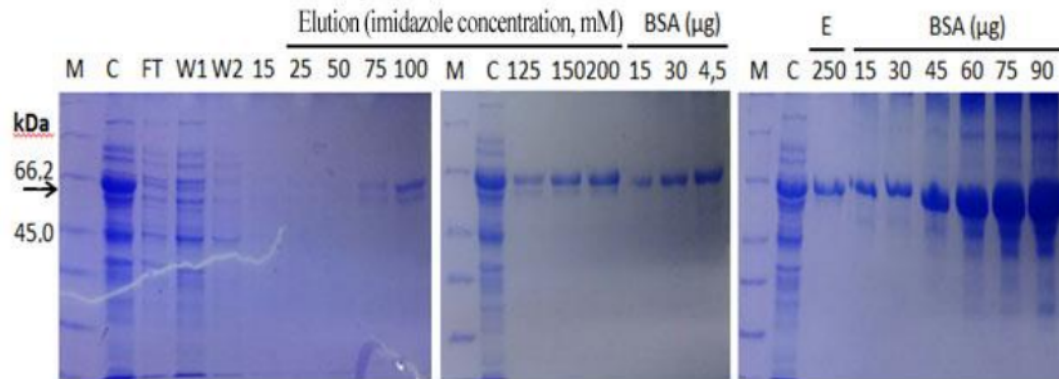


Figure 5. SDS-PAGE of purified *MerA* protein using different concentration of imidazole. (M) maker, (BSA) bovine serum albumine.

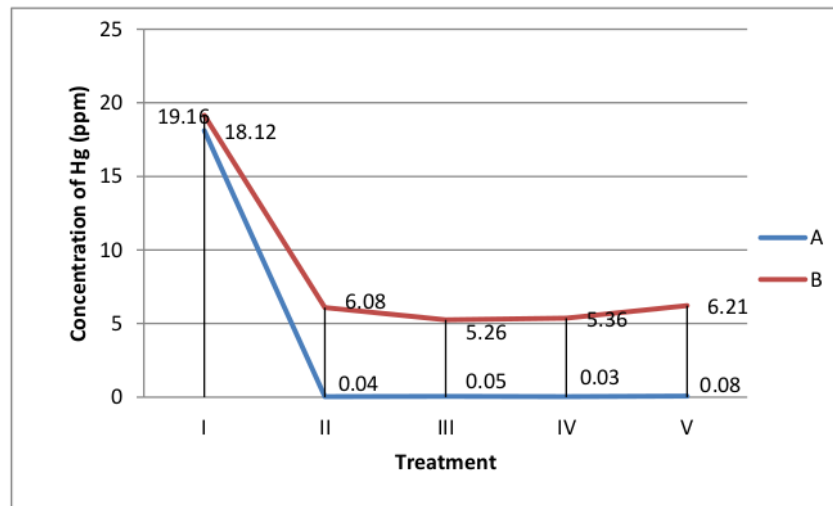


Figure 6. Profile of inorganic mercury reduction by recombinant *E. coli* BL21 (DE3), resulted from *merA* transformation (A) and overproduced mercuric reductase *MerA* (B).

## DISCUSSION

Mercuric reductase is a central enzyme in the mercury compound resistance system by bacteria. The *merA* gene encodes for mercuric reductase enzyme, an enzyme that plays a role in the reduction of highly toxic ionic  $Hg^{2+}$  into nontoxic volatile metallic  $Hg^0$  species (Nascimento and Chartone-Souza, 2003). This gene presents in operon system called *mer operon* contained on bacterial plasmid (Ravel et al., 2000; Nascimento and Chartone-Souza, 2003) and on bacterial chromosomal DNA (Osborn et al., 1997).

This study proves that recombinant *MerA*

protein can be produced by *E. coli* BL21. This enzyme can be used in mercury detoxification. Protein expression was high enough and resulted in efficient protein isolation and purification, thus allowing production of mercuric reductase in large scale. The *merA* gene was synthesized based on putative *merA* gene of *K. pneumoniae* isolated from polluted river estuary (Fatimawali et al., 2015). The synthesized gene was successfully transformed into *E. coli* Top-10. As seen in Figure 2, the full *merA* gene was detected on 1% agarose gel with the size of 1695 bp. This result is similar to the the finding of Zeyullah et al. (2010),

Shah and Ali (2010), and Fatimawali et al. (2015). The transformed *E. coli* BL-21 (DE3) could reduce 100% of inorganic mercury  $\text{HgCl}_2$  when incubated at 37°C for 24 hour.

The ability of this bacteria to reduce inorganic mercury is higher than was performed by mercury resistant bacteria found by Fatimawali et al. (2011) which only reduced inorganic mercury up to 99.4% in 24 hours. This may be because recombinant bacteria contain more *MerA* proteins than the Hg-resistant bacterial cells obtained from the environment. The use of bacteria to rehabilitate mercury contaminated environment is one of environmental friendly methods (Shah and Ali, 2010).

Zeyaulah et al. (2010) explained that expression of bacterial *merA* gene had been expressed in plants but none of them have been already applied in the field and remains being debated. Although *MerA* alone can detoxify mercury enzymatically, *MerB* is also required to achieve higher level of mercury detoxification. The *MerA* protein is limited to enzymatic detoxification of inorganic mercury compounds therefore *MerB* protein is needed to complement the detoxification process since it transforms both organic and inorganic mercury into volatile forms (Mathema et al, 2011).

The overproduced of *MerA* protein had a size of approximately 63kDa, as detected on SDS-PAGE (Figure 4). This result is similar to 66.2 kDa *MerA* protein found by Zeyaulah et al. (2010). The overproduction was induced by IPTG, the common inducer in increasing the expression of recombinant protein. The addition of IPTG needed to be optimized since this chemical can affect the the amount of protein produced. The result showed that optimum overproduction of *MerA* protein was induced by IPTG 0.1 mM. This is in line with the result of Fatimawali et al. (2015). However, Zeyaulah et al. (2010) found that the optimum IPTG was 1 M.

In the reduction test of recombinant *MerA* protein, FAD and NADH were used as cofactor and the source of electron to help the reduction process of inorganic mercury by *MerA* enzyme. The result showed that the ability of *MerA* protein to reduce  $\text{HgCl}_2$  was 80% in the solution. Although it was still less than the reduction power of *MerA* protein produced by recombinant *E. coli* BL21 (DE3) which had a reduction power up to 100% in the solution, however, our finding provides the information of using *MerA* enzyme in enzymatically remediation of polluted environment caused by inorganic mercury. Essentially, the

<sup>11</sup> *MerA* enzyme converts toxic, soluble and bioavailable  $\text{Hg}^{2+}$  into insoluble metallic mercury, which in its reduced metallic state is essentially nontoxic. The possibilities of factors that influence enzymatic reduction power of *MerA* protein needs to be considered by optimization of several factors including pH, temperature, the time of incubation, and the compounds used as electron source.

## CONCLUSION

Transformation of *merA* gene and overproduction of *MerA* protein were successfully performed in *Escherichia coli* BL21 mediated by plasmid pJExpress 414, resulting *merA* gene with nucleotide sequence of 1695 pb and protein with a molecular weight of 63 kDa. The overproduction was executed at 37°C for 4 hours and in 0.1 mM IPTG. The overproduced *MerA* protein reduces 80% of 10 ppm  $\text{HgCl}_2$  in medium with the pH of 7.4 at 37°C for 24 hours, while recombinant *MerA* in *E. coli* BL-21 (DE3) reduced 100% of 10 ppm  $\text{HgCl}_2$  under the same condition. Therefore, overproduced *MerA* protein can be used in the enzymatic detoxification of inorganic mercury.

## <sup>23</sup> CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

## ACKNOWLEDGEMENT

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## AUTHOR CONTRIBUTIONS

All authors contributed to the research and manuscript preparation. F performed the research in the laboratory. BK did the data analysis. F, BK, <sup>29</sup> and TET prepared the manuscript and its revision. All authors read and approved the final version.

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